DESIGNING DISULFIDE CYCLIC PEPTIDE AS FUSION INHIBITOR THAT TARGETS DENV ENVELOPE PROTEIN

Usman Sumo Friend Tambunan[a]*, William Chua[a], Arli Aditya Parikesit[a], Djati Kerami[b]

[a]Bioinformatics Research Group, Department of Chemistry, Faculty of Mathematics and Science, Universitas Indonesia, Depok 16424, Indonesia
[b]Mathematics Computation Group, Department of Mathematics, Faculty of Mathematics and Science, Universitas Indonesia, Depok 16424, Indonesia

Graphical abstract
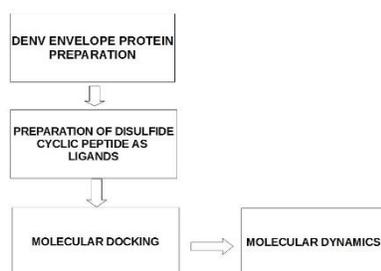
Abstract

Dengue has been a major health concern and currently there is no available option to treat the infection. It is an arboviral disease caused by dengue virus (DENV), an enveloped flavivirus. DENV initiates fusion process between viral envelope and host cell membrane, transfers its viral genome into target cell and infects host. Our research is focused on designing disulfide cyclic peptides that can fit into fusion cavity and interact with fusion peptide, interrupt conformational changes and therefore inhibit the fusion process. Computational approaches were conducted to calculate the binding affinity and stability of disulfide cyclic peptide ligands with target DENV E glycoprotein. Molecular docking and molecular dynamics simulation were performed using Molecular Operating Environment 2008.10 software (MOE 2008.10). Screening of 1320 designed ligands resulted in 3 best ligands, CLREC, CYREC and CYREC that can form interaction with target cavity and peptide fusion. These ligands showed good affinity with target DENV E glycoprotein based on free binding energy and interactions. To evaluate protein-ligand stability, we performed molecular dynamic simulation. Only CLREC showed protein-ligand stability and maintained interaction between ligand and target cavity. Therefore we propose CLREC as potential DENV fusion inhibitor candidates.

Keywords: Dengue, disulfide cyclic peptide, molecular docking, molecular dynamics, fusion inhibitor

1.0 INTRODUCTION

Dengue is a viral infection transmitted by Aedes mosquitoes. Dengue is a flavivirus with five different antigenically distinct serotypes [1]. There are currently no available drugs nor vaccine for dengue. Invention of dengue vaccine is a major step to cope with this disease. Infection by different serotypes trigger condition known as Antibody Dependent Enhancement (ADE), therefore developed vaccine must offer protection against all dengue serotypes. In addition to vaccination, successful vector control would be a useful adjunct to control dengue [2].

DENV particle has diameter about 50-60 nm, consist of an outer glycoprotein shell and an internal host derived lipid bilayer. Within this bilayer is an RNA–protein core consisting of genome RNA and capsid proteins (C). The viral genome consists of a positive-

sense RNA of 11 kb. This RNA encodes three structural proteins (C, prM and E) that form the components of the virion, and seven nonstructural proteins (NS1, NS2A/B, NS3, NS4A/B, NS5) involved in viral RNA replication [3].

As obligatory intracellular parasites, viruses must transfer their genetic information across cellular membranes to infect patient and produces progeny virions. For enveloped viruses, this process requires merging of the viral envelope with target cell membranes and formation of a fusion pore through which the genome of the particle is released. Membrane fusion for viral entry is mediated by membrane protein complexes. These envelope glycoproteins are typically observed as characteristic protein 'spikes' on the surface of purified virions in electron microscopic images. Two basic mechanistic principles of membrane fusion known are target membrane engagement and refolding into hairpin-like structures. Firstly, upon particle attachment to target cells, conformational rearrangements of the fusogenic spike protein complex take place that result in the insertion of largely hydrophobic membrane attack domains, termed the fusion peptides or fusion loops, into the target membrane. The resulting prehairpin intermediate conformation thus engages both the viral envelope and the host cell membrane. Secondly, refolding of the trimeric fusogenic complex onto itself into a trimeric hairpin structure that is considered to bring the attack and transmembrane domains, and thus the donor and target membranes, into close proximity [4,5].

The dengue virus surface is composed of 180 copies of envelope glycoprotein and membrane protein. The immature dengue virus particle is covered with 60 asymmetric trimers of prM-E heterodimers that stick out like spikes from its surface. In the mature virus, the E proteins exist as homodimers that lie on the viral membrane in the form of 30 so-called "rafts". Each raft contains three parallel dimers arranged in icosahedral symmetry and organized into a herringbone pattern. The structural transitions from immature ('spiky') to mature ('smooth') occur while in transit through the Trans-Golgi Network (TGN) and are driven predominantly by conformational changes in the E protein. These conformational changes in E are triggered by low pH (5.8–6.0). The immature DENV particle could exist reversibly in either 'spiky' or 'smooth' forms depending on the pH of the cellular environment. The structure of soluble E protein elucidated by X-ray crystallography consists of three domains: domain I, the N-terminal part structurally located in the central part; domain II, the fusion domain containing a hydrophobic fusion peptide; domain III, the putative receptor binding. During endocytosis, under the acid condition in endosome, the E proteins undergo a dramatic structural change from dimer into trimer. These trimers cluster on the viral surface and induce curvature that might promote fusion. In the E trimer, the fusion peptide is exposed at the tip of the trimer, leading the virus and endosomal membranes to merge [6]. A greater understanding of dengue virus biology has meant that targets within the lifecycle have been identified that could potentially be the site of a therapeutic agent. One potential mechanism of action of an anti-dengue drug is through inhibition of viral entry. The fusion of the viral membrane with the host membrane is mediated by dengue virus E protein [2].

Entry of enveloped viruses into host cell is inititated when virus particle attached itself to host cell receptor and followed by fusion between virus E protein and host cell membrane. Viruses enter host cell through endocytosis and low pH in endosome triggers conformational changes from dimer into monomer and from monomer into homotrimer. During transition from dimer to trimer, residue 83-100 acts as flexible zone (hinge region) which enables rotation and movement of E protein. This conformational changes caused fusion peptide to interact with target membrane, initiates fusion and merges host cell with virus [7,8].

Yennamalli et al., in 2009, proposed novel cavity (II) in DENV-2 envelope protein which formed by 33 residues: residue 1–8, 28, 30, 44, 151–155 and 316 from chain A, with residue 97–109 and 244–247 from chain B [8]. This site has volume of 298 $\mathring{A}^3$ and solvent accessible area of 1190 $\mathring{A}^2$. This site is only available in dimer form and undergoes conformational changes when forming trimer. This site is also near to fusion peptide (AA 98-111)[9] and three hydrophobic amino acid residues (W101, L107 and F108) which have important role in fusion peptide activity are residues that constitutes this cavity [10]. Therefore, this cavity is a potential target to inhibit DENV life cycle.

The role of stabilizing the protein structure and folding was conducted by cyclization via a disulfide bridge [11]. The stabilization was reached by the decrease of entropy and a robust local interaction, that was commonly utilized for designing bis-cystine based cyclic peptides [12]. Moreover, the intermolecular disulfide linkage may be formed if peptides containing more than one cystein residue was available [11]. It may happen due high dilution, and the oxidation was done under basic conditions. In this end, we seek for a feasible alternative in cyclic peptide design, as inhibition activity could be obtained from peptides derived from the stem of dengue-virus E [13]. We argue that the thiol containing compounds could be possibly protected from oxidation/reduction cycles that potentially destroying the intended chemical and 3D structures of the drug, based upon the previous cyclic peptide research of our group [14–16].

In this research, computational approach was performed to design and screening of disulfide cyclic peptides as DENV fusion inhibitor candidates. Purpose of this research is to design disulfide cyclic peptide which can interact with Cavity II in DENV envelope protein. Ligand that binds with cavity can stabilize dimer or prevent transition of protein dimer into trimer, therefor fusion process can be inhibited. The design of these cyclic peptide ligands which can interact with target cavity may be utilized as antiviral candidate to

inhibit fusion between DENV envelopes and host cell membrane.

2.0 EXPERIMENTAL

In this research, we were using Intel Core i3 3,20 GHz processor which is supported with NVDIA CUDA 1024 MB, 128 bit VGA card. Operating system used was Microsoft Windows 7 Ultimate to run multiple online and offline modeling software and simulation [17–20].

2.1 DENV Envelope Protein Preparation

DENV E protein sequences in FASTA format were downloaded from NCBI (National Center of Biotechnology Information) database which can be accessed at http://www.ncbi.nlm.nih.gov/. Sequence alignment of DENV E protein was performed using online ClustalW2 program which were accessed at http://www.ebi.ac.uk/Tools/services/web_clustalw2/toolform.ebi.

Result from alignment was then used as input data for homology modeling. Homology modeling was conducted using Swiss-Model Workspace (http://swissmodel.expasy.org/), so that we got three dimensional DENV E protein template. We searched for protein structure at Protein Data Bank (PDB) through http://www.rcsb.org/pdb/ based on DENV E protein template from previous result.

Three dimensional structure of DENV E protein and target cavity were visualized using Discovery Studio 3.1 clients' software. Geometry optimization and energy minimization were performed by eliminating water molecule, protonation, addition of hydrogen atom and partial charge adjustment. Protonate3D, hydrogen fix and partial charge option were executed respectively. Parameter used was current force field. Energy minimization were using force field, gas phase solvation until RMS gradient 0.05 kcal/Å mol. Default value in MOE were applied for simulation.

2.2 Preparation of Disulfide Cyclic Peptide as Ligands

We designed cyclic peptide ligands based on characteristic of target cavity which is hydrophobic and charged, therefore hydrophobic and charged amino acid were selected to construct peptide [21]. Two cysteine residue were then added at both terminal and cyclized to form disulfide bridge [8]. Several ligands from previous researches were used as control in this research [8,22–24].

Designing and three dimensional modeling were conducted using ChemBioOffice 2010 and Vega ZZ. Ligand geometry optimization were performed using MOE 2008.10 software. Wash option were chosen and partial charge was adjusted for each ligand. MMFF94x forcefield and gas phase solvation were applied in optimizing ligands. Energy minimization was performed until RMS gradient 0,001 kcal/ Å mol.

2.3 Molecular Docking

Molecular docking was performed by choosing MOE-dock option. Triangle matcher [25] was generated as placement method with the total number of rotation was 2.5×10^6. Scoring function used was London dG to predict affinity based on bonding and non-covalent interaction between ligand and receptor [26,27]. During refinement process only 100 pose were retained and only 1 best pose was kept as final docking pose. Identification of interaction between protein – ligand was performed using LigX – interaction in MOE 2008.10 software. Visualization of docked protein – ligand complex were using Discovery Studio 3.1 clients' software.

2.4 Molecular Dynamics

Molecular dynamics simulation was carried out at two different temperatures, 309K and 312K. Born solvation was chosen and RMS gradient was set to 0.05 kcal / Å mol. Geometry optimization and energy minimization of protein - ligand were performed. The ensemble parameter was canonical NVT ensemble (N number atoms, V volume, T temperature), NPA algorithm, and Cutoff restraint of 6 Å. Dynamics simulation stage involved initialization for 100 ps and main simulation until equilibrium state had been reached and cooling stage to obtain trajectory.

Following [28–30], molecular dynamics simulation of complex enzyme-ligand at temperature of 309 K and 312 K was conducted by heating protein - ligand complex for 20 ps, main simulation was employed for 5000 ps and cooling for 20 ps. Position, velocity and acceleration were saved every 0.5 ps, other parameters were set to default value in MOE 2008.10.

3.0 RESULTS

The supplementary material of this research could be accessed here: http://staff.ui.ac.id/system/files/users/aditya.parikesit/material/supplementary_materials_william_0.pdf

3.1 DENV Envelope Protein Preparation

From 309 sequences downloaded at NCBI database and after sequence alignment, we got 147 asterisk (*) symbol which shows identical residue among all sequences, 84 (:) symbol and 43 full stop (.) symbol which represent conserved region [31]. DENV envelope protein with Genbank code AAY34763.1 was selected as representative which has good homology score.

Result from Swiss-Model Workspace (http://swissmodel.expasy.org/) showed protein structure which has the highest homology with input sequence. Protein with PDB code 3G7T (homology 92%) was not used because the structure was in post fusion state. Protein with PDB code 1UZG (homology

77%) were also not used because of differences in residue which constructs the target cavity. Protein with PDB code 1OKE showed 67% homology was chosen in this research but the identical 1OAN was used in simulation in accordance to research by Yennamalli et al., in 2009, which found cavity in this structure. DENV E protein with PDB code 1OAN is 100% identical to 1OKE. Superpose between 1OAN and 1OKE showed RMSD (Root Mean Square Deviation) equals to 1,102 Å. Two or more protein were categorized as homologs if RMSD value were ≤ 3 Å. Therefore 1OAN is homolog to 1OKE (Figure 1 of supplementary material).

Research by Yennamalli et al., in 2009 showed cavity which can be occupied by ligand and affects conformation of DENV envelope protein [8]. This cavity was composed of 33 amino acid residue: residue 1–8, 28, 30, 44, 151–155 and 316 from chain A, with residue 97–109 and 244–247 from chain B. Using Discovery Studio 3.1 clients, visualization of this cavity was shown in Figure 2 of supplementary material.

3.2 Preparation of Disulfide Cyclic Peptide as Ligands

Combination of 10 amino acid residue which has hydrophobic side chain (alanine, phenylalanine, glysine, isuleucyne, leucyne, methionine, proline, tryptophan, tyrosine and valine), 3 positive charged amino acid (arginine, hystidine and lysine), also 2 amino acid with negatively charged side chain (aspartic acid and glutamic acid) resulted in 1320 disulfide cyclic peptide consist of tripeptide, tetrapeptide and pentapeptide.

3.3 Molecular Docking

Molecular docking is a method to predict conformation and orientation of ligand towards target structure. Ligand conformation were adjusted by placing ligand in different orientation onto receptor to get most suitable and actual receptor-ligand binding mode. Scoring function was used to calculate energy of each receptor-ligand interaction pose. Result score give us prediction of interaction and biological activity of ligand toward receptor [32].

Screening of 1320 ligand resulted in 3 best ligand based on free energy binding, protein-ligand interaction and Lipinski's Rule of Five.

Thermodynamically, intermolecular interaction and protein-ligand complex formation was affected by changes in Gibbs free energy (ΔG). Negative Gibbs energy shows spontaneous reaction [27]. From Table 1, CLREC, CFREC and CYREC showed lower (negative) ΔS or ΔG value compared to control, these showed that affinity between ligands and cavity were better than control ligands.

Table 1 Free binding energy in molecular docking

No.	Ligand	ΔG (kcal/mol)
1.	1071 CLREC	-30,7284
2.	1171 CYREC	-30,3522
3.	1121 CFREC	-28,8963
4.	C6 standard Wang	-19,4870
5.	NITD448 standard Poh	-19,3088
6.	A5 standard Kampmann	-18,4387
7.	R1 standard Yennamali	-15,0584
8.	A4 standard Kampmann	-13,6520

Table 2 showed that CLREC formed 5 hydrogen bond, CYREC formed 6 hydrogen bond and CFREC formed 5 hydrogen with cavity. As for control ligands, NITD448 standard Poh, R1 standard Yennamali and A4 standard Kampmann formed 4,1 and 1 hydrogen bond with cavity respectively. Hydrogen bond between protein-ligand can reduce E protein flexibility and disturbance in conformational changes.

Table 2 Protein-ligand hydrogen interaction

No.	Ligand	Hydrogen Bond
1.	1071 CLREC	Ser72 (2), **Gly102** (2), **Asp154** (1), **Arg2** (1), **Asn103** (2), **Lys246** (2)
2.	1171 CYREC	Ser72 (2), **Gly102** (2), **Asp154** (1), **Lys246** (2), **Arg2** (1), **Arg99** (2), **Asn103** (2)
3.	1121 CFREC	Ser72 (2), **Gly102** (2), **Asp154** (1), **Thr155** (1), **Arg2** (1), **Lys246** (1)
4.	C6 standard Wang	---
5.	NITD448 standard Poh	**Arg2** (1), Ser72 (2), **Gly102** (2), **Asn103** (2), **Lys246** (2)
6.	A5 standard Kampmann	---
7.	R1 standard Yennamali	**Gly 102**(1)
8.	A4 standard Kampmann	**Arg99** (2)

Note: Bold letter shows residue that construct cavity, Red color shows fusion peptide residue. (1) Chain A of DENV E protein dimer. (2) Chain B of DENV E protein dimer.

Visualization of binding pose for CLREC, CYREC and CFREC were shown in Figure 1. According to Lipinski's Rule of Five, a good drug must have molecular weight range between 500 g/mol, H-donor < 5, H-acceptor < 10, and log P < 5. Molecular weight showed the drug size. H-donor and H-acceptor describes probability for drug interaction. These parameter determines permeability of drug molecules through bilayer cell membrane. Log P was related to hydrophobicity of drug molecule. Increase in logP shows increase in hydophobicity [33,34].
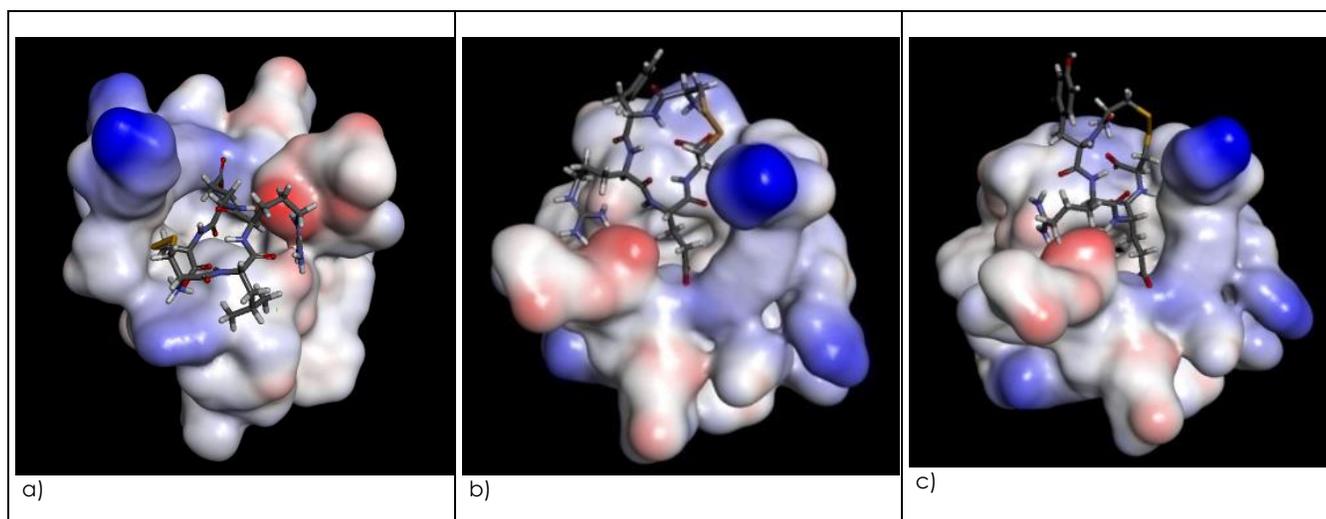
Figure 1 Visualization of binding pose between disulfide cyclic peptide and target cavity. A) CLREC B) CYREC C)CFREC

Table 3 showed that proposed ligands has similar hydrophilic properties. Ligand has low permeability through lipid bilayer and H donor that is more than 5 may influences ligand's affinity to target receptor and affect ADMET properties of ligands.

Table 3 Lipinski's rule of five analysis for disulfide cyclic peptide ligands

Ligand Parameter	CLREC	CYREC	CFREC
Mol.weight (g/mol)	620,753	654,770	670,769
H-*donor*	8	8	9
H-*acceptor*	8	8	9
LogP	-7,299	-7,103	-7,397

Note: Red color showed disagreement with Lipinski's Rule of Five.

3.4 Molecular Dynamics

Solvation effect was observed in dynamics simulation. Interaction between protein-ligand in solvent model was evaluated at certain duration. The time evolution of the hydrogen bonds from the inhibitor-enzyme complex provided an approach to evaluate the convergence of the dynamical properties of the system. Result data of protein-ligand interaction during simulation at 309K (healthy people body temperature) was shown in Table 4 of supplementary material.

From Table 4, we can see that ligand CLREC and CYREC maintained interaction with target cavity by the end of simulation. Only CLREC showed hydrogen interaction with fusion peptide segment during simulation for 5000ps. Interaction between ligand and target cavity will reduce movement of envelope during conformational changes and based on research by Melo M.N., in 2009, Trp101, Leu107 and Phe108 has important roles in membrane fusogenic

activity, interaction between ligand with Trp101 as showed in table describes possibility that fusion peptide activity will be affected because of interaction with CLREC.

Molecular dynamics at 312 K also showed changes in protein-ligand interaction showed in Table 5 of supplementary material.

CLREC and CYREC maintained interaction with target cavity until the end of simulation, while CFREC lost interaction with target after simulation 4000ps. At simulation 312K, CLREC also maintained interaction with fusion peptide segment inside cavity but did not form any interaction with three important residue W101, L107 and F108.

Conformational changes of protein-ligand complex was shown in calibration curve between simulation times versus Root Mean Square Deviation (RMSD). Protein conformation is a series of three dimensional coordinate. In molecular dynamics, movement of atoms at each trajectory caused conformation changes of protein. Value of RMSD shows degree of difference between these states.

Figure 2 shows correlation plot between simulation time and RMSD value. From these plot, it can be seen that protein-ligand complex in solvated system showed dynamic changes. Fluctuation of RMSD value describes the tendency of protein-ligand system to find the most suitable geometry for protein-ligand complex. We can see from the graph that the pattern of correlation plot between simulation at 309K and 312K is similar. Protein-ligand complex CLREC and CYREC is quite stable because RMSD fluctuation reached constant value after simulation 3000ps – 3500ps. As for CFREC, RMSD value was still fluctuated meaning that complex was still searching for more stable conformation.
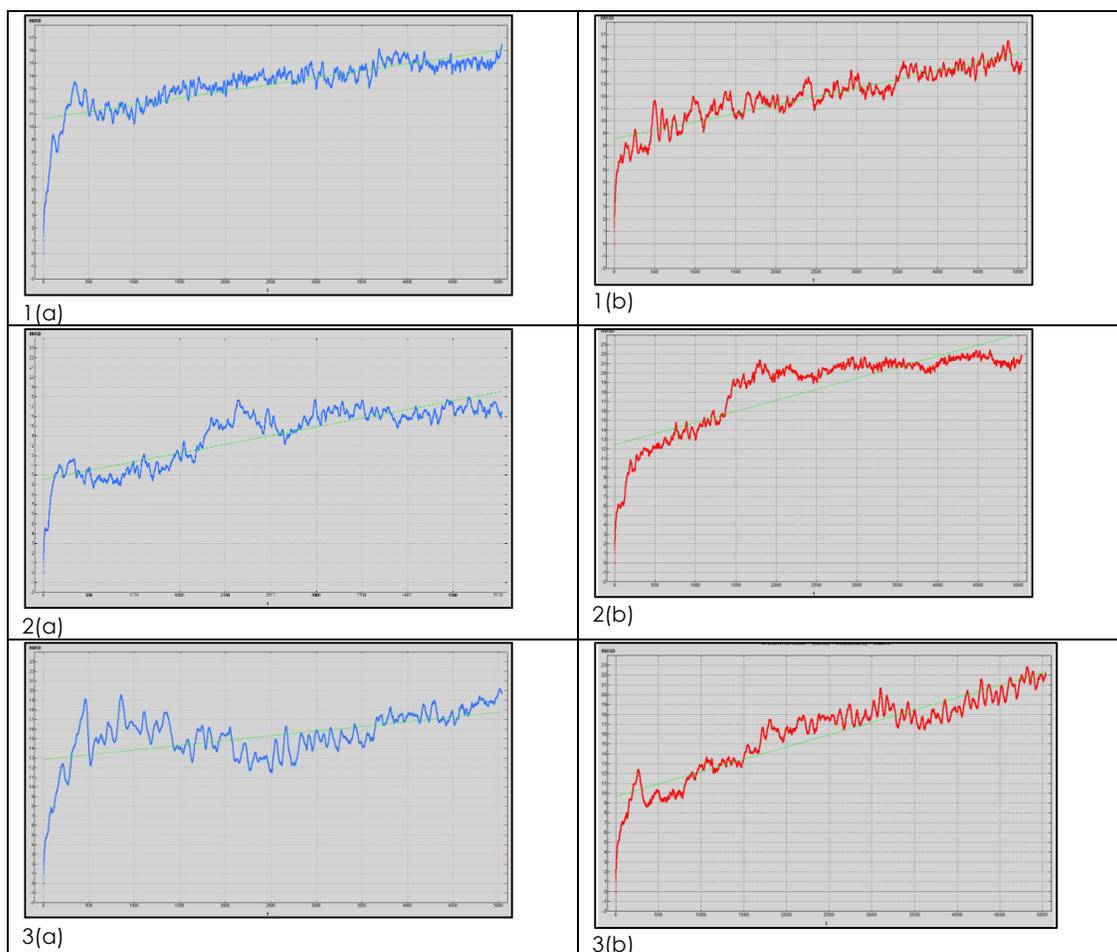
Figure 2 Correlation plot of simulation time vs RMSD. 1(a) ligand CLREC (309 K); 1(b) ligand CLREC (312 K); 2(a) ligand CYREC (309 K); 2(b) ligand CYREC (312 K); 3(a) ligand CFREC (309 K); 3(b) ligan CFREC (312 K)

Both CLREC and CYREC maintained interaction with target cavity and formed stable protein-ligand complex during simulation for 5000ps but CLREC has better affinity with target cavity based on interaction with target cavity.

Visualization of protein-ligand binding pose after molecular dynamics simulation was shown in Figure 3. We can see that at both temperature 309K and 312K, only CLREC was still located at target cavity and formed interaction with residue that builds cavity. Ligand CYREC formed interaction with target cavity but tends to leave cavity site. Ligand CFREC had left cavity site and formed interaction with residue that is near to cavity site but did not build up target cavity. Therefore CLREC was proposed as better candidate for fusion inhibitor.
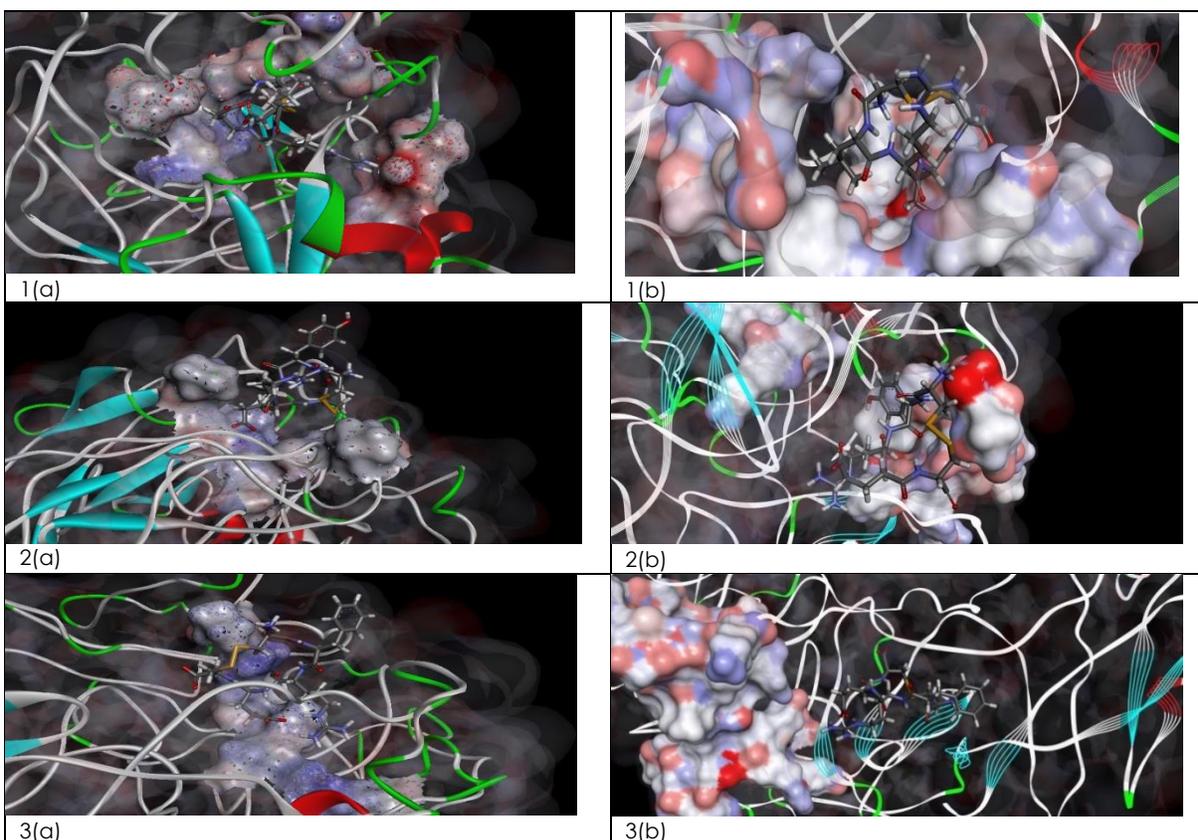
Figure 3 3D visualization of protein-ligand binding pose. 1(a) ligand CLREC (309 K); 1(b) ligand CLREC (312 K); 2(a) ligand CYREC (309 K); 2(b) ligand CYREC (312 K); 3(a) ligand CFREC (309 K); 3(b) ligand CFREC (312 K)

4.0 DISCUSSION

Although Table 3 shows that there are some undisputable hindrance concerning the ligand's observance toward the Lipinsky's rule, we could suggest some possible solution for them. The drug delivery of the ligand toward the targeted cell could be proposed based on pharmaceutical chemistry techniques, such as constructing enzyme inhibitors to slow down the rate of the protease enzyme, absorption enhancers, and chemical modifications [35] . Another possible solution is the construction of prodrugs that enhances the durability of the drugs toward the targeted receptors [36]. Indeed, as the pharmaceutical technologies are progressing forward, the drug delivery issues will eventually be able to be resolved. In our work, we argued that the RMSD resolution of molecular dynamics is much improved than the previous ones. We also drawn a more fine grained annotation of the enzyme's active site than the old method [21]. This research is not using commercial cyclic peptide database as our previous work, instead we developed the most fit peptide sequence as inhibitor.

5.0 CONCLUSION

We had designed 5 tripeptide, 65 tetrapeptide and 1250 pentapeptide ligands by combining hydrophobic and charged amino acid residue which was made into cyclic by adding cysteine residue at each terminal to form Disulfide Bridge. We performed molecular docking simulation and got three best ligand after screening of 1320 ligands. These ligands showed lowest (negative) ΔG and good affinity with target cavity beneath DENV E protein compared to control ligands from previous researches. CLREC, CYREC and CFREC formed hydrogen bond with residue that build up target cavity while molecular docking simulation. But in molecular dynamics simulation, only CLREC and CYREC maintained interaction after simulation for 5000ps at both temperature 309K and 312K. CFREC did not form interaction with residues that build up target cavity after simulation for 5000ps. Both CLREC and CYREC showed stable conformation based on RMSD value but CLREC has better affinity with target cavity compared to CYREC. We proposed CLREC as potential candidate for DENV E protein fusion inhibitor.

Acknowledgement

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